American Fern Journal 102(1):55–68 (2012)

Molecular Evidence on the Origin of *Osmunda* ×*mildei* (Osmundaceae)

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Abstract.—The southern Chinese *Osmunda* ×*mildei* has been suggested to be an intersubgeneric hybrid, i.e., *O. japonica* (subgenus *Osmunda*) × *O. angustifolia* (subgenus *Plenasium*) or *O. japonica* × *O. vachellii* (subgenus *Plenasium*). These interpretations were based on morphological, cytological, and/or chloroplast DNA data, yet the parents of the hybrid remained unclear. Molecular phylogenetic relationships inferred here from chloroplast *rbcL* sequences and three nuclear DNA markers show that *O.* ×*mildei* is most likely a hybrid between the paternal *O. japonica* and the maternal *O. vachellii*.

Key Words.—EST, intersubgeneric hybrid, *Osmunda japonica*, *Osmunda* ×*mildei*, *Osmunda vachellii*, *rbcL*

The genus *Osmunda* of the leptosporangiate fern family Osmundaceae has natural hybrids (Kato, 2009; Tsutsumi *et al.*, 2011). One such is *Osmunda* ×*mildei* C.Chr. (=*O. bipinnata* Hook., a later homonym of *O. bipinnata* L.), which nearly became extinct in its known range in Hong Kong. However, this hybrid was recently found in Shenzhen, Guangdong, and Mt. Qiyun, Jiangxi (Zhang *et al.*, 2008), and less than 10 individuals are known. It was also found in Zhangjiajie, Hunan (Y.-H. Yan, pers. comm.). It can propagate via spores in experimental conditions (J.-F. Yang, unpubl. data), but it is uncertain if the individuals were derived from spore propagations or from independent formations of the hybrid. *Osmunda* ×*mildei* is characterized by subcoriaceous, bipinnate-bipinnatifid leaves with round, entire pinnules, and fertile pinnae inserted below the middle of the leaf. For the origin of *O.* ×*mildei*, two possibilities were proposed (Fig. 1). Based on karyological and morphological analyses, He *et al.* (2006) suggested that *O.* ×*mildei* is a hybrid of *O. japonica* Thunb. (subgenus *Osmunda*) and *O. angustifolia* Ching (subgenus *Plenasium*). Zhang *et al.* (2008) observed the absence of chromosome pairings at meiosis and resulting abortive spores in *O.* ×*mildei*, and suggested that it is a sterile F1 hybrid, but argued that the parents were *O.*

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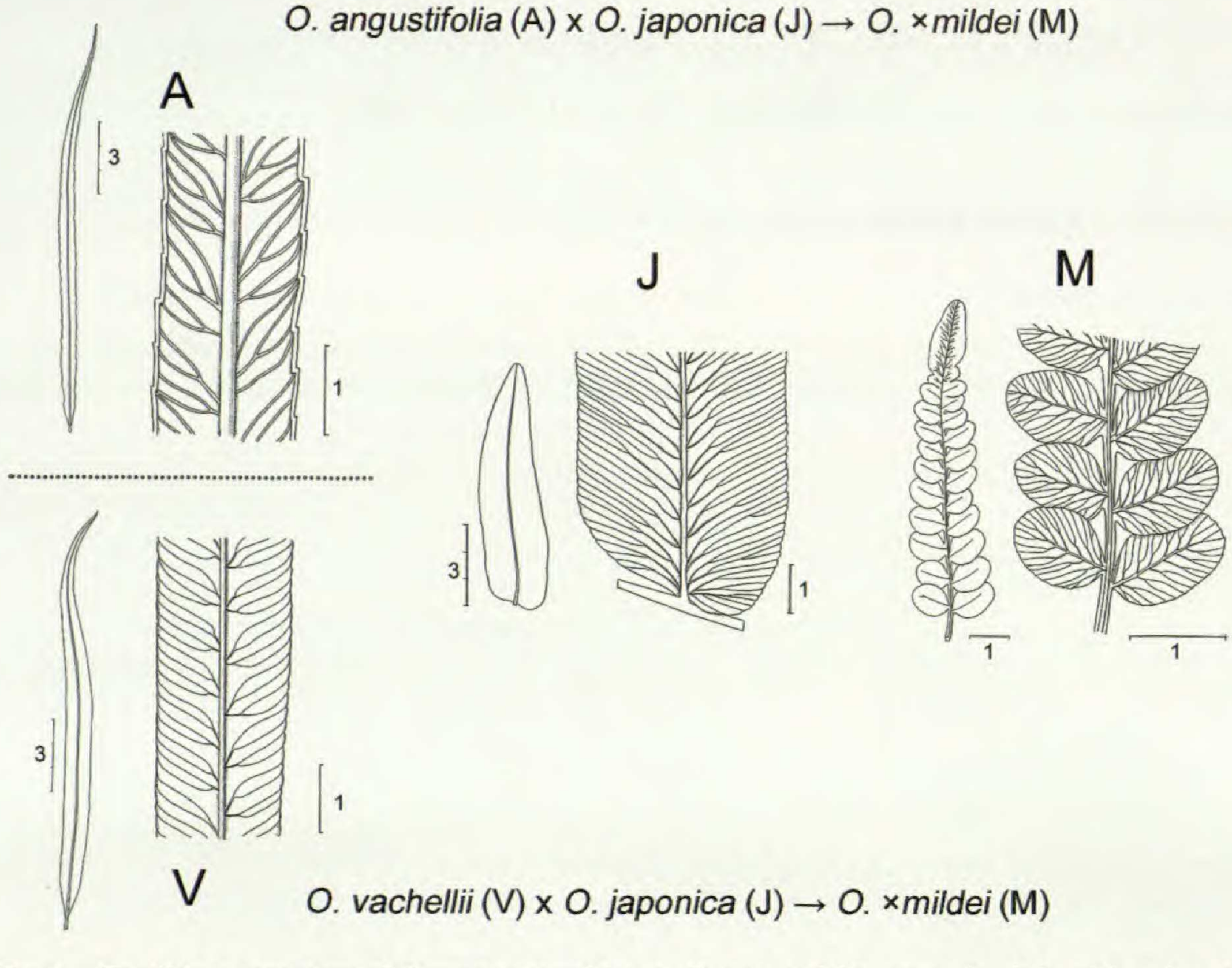

FIG. 1. Candidate parentage of *Osmunda* ×*mildei*. A: *O. angustifolia*: pinna (on left side) and part (on right side). J: *O. japonica*: pinnule and part. M: *O.* ×*mildei*: pinna and part. V: *O. vachellii*: pinna and part. Scale unit is cm. Two proposed crossings are illustrated at the top and bottom.

japonica and *O. vachellii* Hook. (subgenus *Plenasium*), because *O. vachellii* co-occurs with *O.* ×*mildei*, but *O. angustifolia* does not occur in some of the localities of *O.* ×*mildei* (Fig. 2). Gou *et al.* (2008) also proposed that *O. vachellii* is the maternal progenitor of *O.* ×*mildei*, based on inferences from chloroplast DNA sequence data. Under either parentage hypothesis *O.* ×*mildei* is likely an intersubgeneric hybrid between the subgenera *Osmunda* and *Plenasium*.

There are three more known hybrids reported in Osmundaceae (Kato, 2009). Eastern North American *O.* ×*ruggii* Tryon is *O. regalis* L. (subgenus *Osmunda*) × *O. claytoniana* L. (subgenus *Claytosmunda*) (Tryon, 1940; Wagner *et al.*, 1978; Whetstone and Atkinson, 1993; Li and Haufler, 1994). Japanese *O.* ×*nipponica* Makino is *O. japonica* (subgenus *Osmunda*) × *O. claytoniana* (subgenus *Claytosmunda*) (Ito, 1964), but Sugimoto (1979) suggested that it is an intergeneric hybrid, i.e., *O. japonica* × *Osmundastrum cinnamomeum* (L.) C.Presl. Neither is given molecular evidence. Finally, Japanese *O.* ×*intermedia* (Honda) Sugim. is *O. japonica* × *O. lancea* Thunb. (both in subgenus *Osmunda*; Tagawa, 1959; Iwatsuki, 1995; Tatuno and Yoshida, 1966; Yatabe *et al.*, 2009).

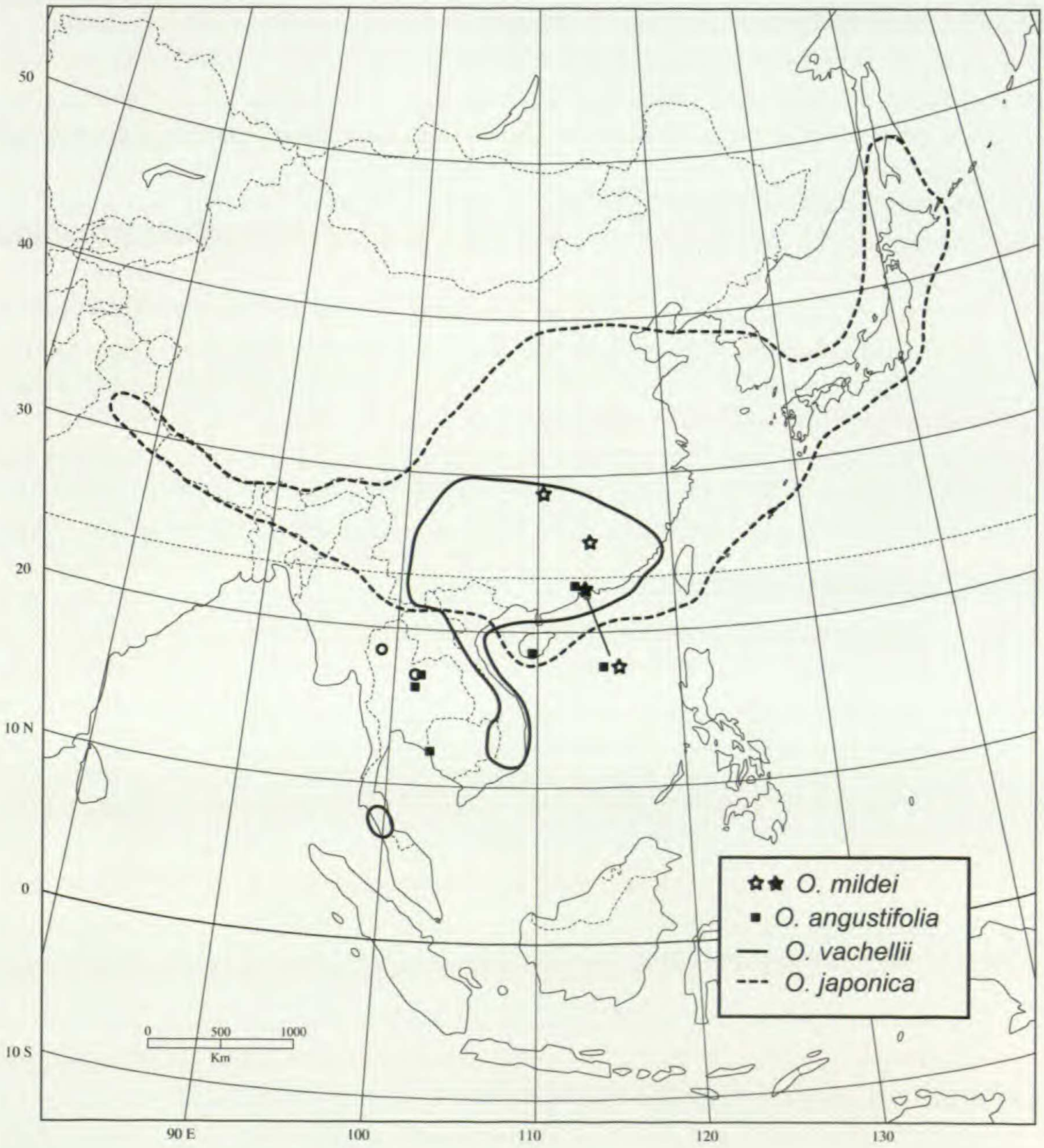

FIG. 2. Map showing distribution of *Osmunda angustifolia* (solid squares), *O. japonica* (broken line), *O.* ×*mildei* (asterisks) and *O. vachellii* (solid line). Solid asterisk shows the place of *O.* ×*mildei* sampled in this study.

The evidence offered to support the parentage of *O.* ×*mildei* in previous studies is inconclusive, because it lacked nuclear sequence data. Combining nuclear and chloroplast DNA data are useful to identify a maternal and a paternal parent. This study examines the aforementioned alternative origins of the hybrid, based on chloroplast *rbcL* sequences and three nuclear DNA sequences.

MATERIALS AND METHODS

Materials.—Samples of three species of subgenus *Plenasium* (*O. vachellii, O. banksiifolia* (C.Presl) Kuhn, *O. angustifolia*), the putative intersubgeneric

hybrid *O.* ×*mildei*, one species of subgenus *Claytosmunda* (*O. claytoniana*), *Osmundastrum cinnamomeum*, and *Todea barbara* (L.) T.Moore, along with three species of subgenus *Osmunda* (*O. japonica, O. lancea, O. regalis*), were collected in the field or botanical gardens. The sources of materials used are shown in Table 1.

Sequencing of chloroplast and nuclear DNA.—Leaf fragments were used for molecular analysis. DNA was extracted from fresh or silica-gel-dried material using a QIAGEN DNeasy Mini Kit (QIAGEN, Valencia, CA) following the manufacturer's instruction. Three nuclear DNA markers (EST_L058, EST_L110, EST_L258) selected from the expressed sequence tag library developed by Yatabe *et al.* (2009) and the chloroplast locus *rbcL* were analyzed. Primers for amplification and sequencing, and detailed information on the three nuclear markers are shown in Table 2 and Table 3, respectively. PCR was performed using a Perkin-Elmer 9700 DNA thermal cycler (Applied Biosystems, Foster, CA) with *Ex Taq* DNA polymerase (TaKaRa Bio, Tokyo, Japan) and Ampdirect Plus (Shimadzu, Kyoto, Japan) in 35 denaturation, annealing, and elongation cycles (30 sec at 94°C; 30 sec at 50°C for the *rbcL* and 58°C in the three nuclear markers; and 90 sec at 72°C) with a final elongation step (7 min at 72°C). The PCR products were purified with ExoSAP-IT (USB corporation, Cleveland, OH) following the manufacturer's instructions. Sequencing was conducted using an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). The raw sequence data were assembled using Seqman II (Dnastar, Madison, WI). For the sample of *O.* ×*mildei*, PCR of the nuclear DNA markers was performed with PrimeSTAR Max DNA polymerase (TaKaRa Bio) in 35 cycles (10 sec at 98°C, 5 sec at 55°C and 5 sec at 72°C). The PCR products were cloned using a pGEM-T Vector System I (Promega, Madison, WI) and at least 10 clones were sequenced. Minor variants from single clones, presumably sequencing errors, were observed. Therefore a consensus sequence was used for each allele type; the differences between each consensus sequence and the original clones are shown in Table 4. The assembled sequences were aligned by Clustal X program (Thompson *et al.*, 1997) and then aligned manually.

Molecular phylogeny.—Phylogenetic analyses were performed by maximum parsimony (MP) and Bayesian analysis. Registered sequences of Osmundaceae in Genbank were added into the analyses (see Table 1). Maximum parsimony (MP) inference was conducted with PAUP* 4.0b10 (Swofford, 2002). The bases that could not been identified were treated as unknown (N), and gaps were treated as missing data. All characters were equally weighed and heuristic searches were conducted with 1000 random addition replicates involving TBR branch swapping. Bootstrap values were calculated from 1000 pseudoreplicates, each with 100 random additions. For the Bayesian analyses, MrModeltest 2.0 (Nylander, 2004) was used to determine the nucleotide substitution model. Bayesian searches were conducted by MCMC with two independent sets of four chains, each run for ten million generations, sampling every 100 generations by MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The nucleotide model selected was: *rbcL*, GTR + I + G; EST_L058, HKY + I; EST_L110, GTR + I; EST_L258, GTR + I. The program

TABLE 1. Materials used in this study. * shows Genbank accession numbers of each locus.

Genus, subgenus, species	Sample ID	Source and voucher	*rbcL**	EST_L058*	EST_L110*	EST_L258*
O. ×*mildei* C.Chr.		Cult. in Shenzhen Fairylake Botanical Garden (originated from China, Guangdong, Shenzhen)	AB672746	See Table 4	See Table 4	See Table 4
Osmunda* subg. *Claytoniana						
O. claytoniana L.	CL1	Cult. in Tsukuba Botanical Garden (originated from Japan); *C. Tsutsumi s.n.* (TNS)	AB672747	AB672752	AB672790	AB672828
	CL2	China, Yunnan, Gongshan, Chichi; *L.-Y. Kuo 9485* (TAIF)	AB639186	AB672753	AB672791	AB672829
	CLg1	Cult. in Missouri Botanical Garden; *G. Yatskievych 99-0302*	AB024950	-	-	-
	CLg2	Cult. in Botanical Gardens, Nikko, University of Tokyo; *S. Nemoto 99-307*	AB024951	-	-	-
Osmunda* subg. *Osmunda						
O. japonica Thunb.	J1	Cult. in Tsukuba Botanical Garden (originated from Japan); *C. Tsutsumi s.n.* (TNS)	AB494711	AB672754	AB672792	AB672830
	J2	Japan, Miyazaki Pref., Kobayashi; *G. Kokubogata GK9416* (TNS)	AB494714	AB672755	AB672793	AB672831
	J3	Japan, Kagoshima Pref., Amami Island, Mt. Yuwan; *M. Kato et al. s.n.* (TNS:764134)	AB494712	AB672756	AB672794	AB672832
	J4	China, Fujian, Mt. Wuyi; *A. Ebihara s.n.* (TNS:762111)	AB494712	AB672757	AB672795	AB672833
	J5	China, Yunnan, Gongshan, Chichi; *L.-Y. Kuo 9468* (TAIF)	AB639162	AB672758	AB672796	AB672834
	J6	Bhutan, Sha-ngawang; *S. Matsumoto W.G.M.124* (TNS)	AB494711	AB672759	AB672797	AB672835
	J7	India, Meghalaya State, Shillong Peak ridge, by site of ruined "Peak Lodge"; *C. R. Fraser-Jenkins s.n.* (TNS:740377)	AB639163	AB672760	AB672798	AB672836
	J8	India, Central Himalaya, from Fern Garden, Department of Botany, Government Postgraduate College, Pithoragarh, 262502. (Originated from Champawat); *C. R. Fraser-Jenkins s.n.* (TNS:777785)	AB639164	AB672761	AB672799	AB672837
	Jg1	Japan, Shizuoka Pref.: *Y. Yatabe 99-0303*	AB024947	-	-	-
O. lancea Thunb.	L1	Cult. in Tsukuba Botanical Garden (transplanted from Shizuoka Pref.); *C. Tsutsumi s.n.* (TNS)	AB494711	AB672762	AB672800	AB672838
	L2	Japan, Kochi Pref.; *A. Ebihara et al., KC2007-1408* (TNS:766627)	AB494713	AB672763	AB672801	AB672839
	L3	Japan, Miyazaki Pref.; *T. Minamitani s.n.* (TNS)	AB494711	AB672764	AB672802	AB672840
	Lg1	Cult. in Botanical Gardens, Koishikawa, University of Tokyo; *K. Hirai 99-0305*	AB024952	-	-	-

TABLE 1. Continued.

Genus, subgenus, species	Sample ID	Source and voucher	*rbcL**	EST_L058*	EST_L110*	EST_L258*
O. regalis L.	R1	India, Kerala, Kozhikode Dist., Vellarimala; *A. K. Pradeep 73207* (TNS:771612)	AB639165	AB672765	AB672803	AB672841
	R2	India, Karnataka, Shimoga Dist., Kemmanagundi near Shimoga; *J. Murata & H. Murata s.n.* (TI)	AB639166	AB672766	AB672804	AB672842
	R3	India, Kerala, Kozhikode Dist., Vellarimala; *A. K. Pradeep 86941* (TNS)	AB639167	AB672767	AB672805	AB672843
	R4	India, Pachmarhi (cult. in National Botanical Research Institute Botanic Garden); *M. Kato s. n.* (TNS)	AB639168	AB672768	AB672806	AB672844
	R5	Cult. in the home garden of H. P. Nooteboom in Netherlands	AB639169	AB672769	AB672807	AB672845
	R6	Netherlands, 12 km S of Venlo, Limburg prov.; *P. Hovenkamp s.n.* (L)	AB639170	AB672770	AB672808	AB672846
	R7	Germany, Rhineland-Palatinate, Palatinate Forest Biosphere Reserve, 15 km S of Kaiserslautern; *M. Zink s.n.*	AB639171	AB672771	AB672809	AB672847
	R8	Macedonia, Strumica, Kolesino, Orman; *A. Tuji et al. s.n.* (TNS:737424)	AB639172	AB672772	AB672810	AB672848
	R9	Macedonia, Strumica, Kolesino, Orman; *A. Tuji et al. s.n.* (TNS:737429)	AB639173	AB672773	AB672811	AB672849
	R10	Cameroon, Mbalmayo, Ebogo, Nyong River; *M. Kato et al. CMR-141* (TNS)	AB639174	AB672774	AB672812	AB672850
	R11	Madagascar, Perinet, Andasibe; *T. Nakamura s.n.* (TNS)	AB639175	AB672775	AB672813	AB672851
	R12	Madagascar, Perinet, Vohmana; *T. Nakamura s.n.* (TNS)	AB639176	AB672776	AB672814	AB672852
	R13	USA, New York, N of New York City; *R. Moran 8179*	AB639177	AB672777	AB672815	AB672853
	R14	USA, Indiana, Laporte Co., Ambler Flatwoods Nat. Pres.; *G. Yatskievych 07-91*(TNS:771606)	AB639178	AB672778	AB672816	AB672854
	R15	USA, Missouri, Reynolds Co., Maury Pond; *G. Yatskievych 07-16* (TNS:771608)	AB639179	AB672779	AB672817	AB672855
	R16	USA; *L.-Y. Kuo s.n.* (TNS)	AB639180	AB672780	AB672818	-
	R17	Mexico; *R. C. Monica s.n.*	AB639181	AB672781	AB672819	AB672856
	R18	Argentina, Misiones, Monte Carlos, Colonia Guatambu, Bafiado junto al arroyo Caraguatay; *E. I. Meza Torres, et al. No. 1243* (TNS)	AB639182	AB672782	AB672820	AB672857

TABLE 1. Continued.

Genus, subgenus, species	Sample ID	Source and voucher	*rbcL**	EST_L058*	EST_L110*	EST_L258*
	Rg1	Madagascar	AB076258	-	-	-
	Rg2	United Kingdom	AB076259	-	-	-
	Rg3	Cult. in Missouri Botanical Garden; *G. Yatskievych 99-304*	AB024948	-	-	-
Osmunda* subg. *Plenasium						
O. angustifolia Ching		Cult. in Shenzhen Fairylake Botanical Garden	AB672748	AB672783	AB672821	AB672858
O. banksiifolia (C.Presl) Kuhn	B1	Cult. in Tsukuba Botanical Garden (originated from Okinawa Pref., Japan)	AB024956	AB672784	AB672822	AB672859
	Bg1	Japan, Miyazaki Pref.; *K. Hirai 90-559*	AB024956	-	-	-
	Bg2	Japan, Tokyo, Ogasawara; *K. Hirai 92-267*	AB024955	-	-	-
O. javanica Blume		Cult. in UKM, Malaysia; *N. Murakami 98-M12*	AB024953	-	-	-
O. vachellii Hook.	V1	Cult. in Shenzhen Fairylake Botanical Garden	AB672749	AB672785	AB672823	AB672860
	Vg1	Cult. in UKM, Malaysia; *N. Murakami 98-M13*	AB024954	-	-	-
Leptopteris						
L. hymenophyllum (A.Rich.) C.Presl		New Zealand; *M. Ito & T. Asakawa 97Yg09*	AB024957	-	-	-
L. wilksiana (Brack.) Christ		New Caledonia; *M. Ito & T. Asakawa 97Zm06*	AB024958	-	-	-
Osmundastrum						
O. cinnamomeum (L.) C.Presl	CI1	Cult. in Tsukuba Botanical Garden; *C. Tsutsumi s.n.* (TNS)	AB672750	AB672786	AB672824	AB672861
	CIg1	Cult. in Missouri Botanical Garden; *G. Yatsukievych 98-301*	AB024949	-	-	-
	CIg2	Cult. in Botanical Gardens, Nikko, University of Tokyo; *Hasebe 27624* (TI)	D14882	-	-	-
Todea						
Todea barbara (L.) T.Moore	T1	Cult. in Cibodas Botanical Garden; *C. Tsutsumi s.n.* (TNS)	AB672751	AB672787	AB672825	AB672862
	Tg1	Cult. in Cibodas Botanical Garden (originated from New Zealand); *Y. Yatabe 99-0306xs*	AB024959	-	-	-

TABLE 2. Primers used for the analyses of three nuclear markers.

Marker	Primer	Primer sequence	Reference
EST_L058	EST_L058F	ATAAGGTTTCGCCCTCGAAT	Yatabe *et al.* 2009
	EST_L058R	TCTTGCAGTTGCGAGTTCAC	Yatabe *et al.* 2009
EST_L110	EST_L110F (P108)	CATTGCACATCGGAGATGAT	Yatabe *et al.* 2009
	EST_L110R (P107)	CACAGCTGAAACACCCTGAA	Yatabe *et al.* 2009
EST_L258	EST_L258F	TCATGGCGACTGTGAAGAAG	Yatabe *et al.* 2009
	EST_L258R	CGCCCTTTGGATTTACGATA	Yatabe *et al.* 2009

Tracer (Rambaut and Drummond, 2009) was used to check the runs had reached stationarity and effective sample size of all the parameters was high (>100). The first 2.5 million generations before sufficient stationary generations were discarded as burn-in periods and the rest of trees were used to calculate posterior probabilities. *Osmundastrum cinnamomeum* and *Todea barbara* were used as outgroups (Yatabe *et al.*, 1999; Metzgar *et al.*, 2008).

RESULTS

Bayesian and maximum parsimony analyses of each dataset produced congruent topologies (Bayesian consensus trees shown in Figs. 3–6). Maximum parsimony analyses resulted in three shortest trees of a length of 146 steps (CI = 0.77, HI = 0.23, RI = 0.93) for chloroplast *rbcL* (1227 bp) with 101 parsimony-informative characters, 78 shortest trees of a length of 47 steps (CI = 0.89, HI = 0.11, RI = 0.88) for nuclear EST_L058 (198 bp) with 18 parsimony-informative characters, ten shortest MP trees of a length of 125 steps (CI = 0.91, HI = 0.09, RI = 0.91) for nuclear EST_L110 (572 bp) with 57 parsimony-informative characters, and two shortest trees of a length of 122 steps (CI = 0.89, HI = 0.12, RI = 0.94) for nuclear EST_L258 (361 bp) with 59 parsimony-informative characters.

TABLE 3. Total lengths of three nuclear markers, coding and non-coding regions in *Osmunda japonica* (J3 in Table 1), and identified genes with one of the highest E-value (< 0.001) obtained by Blast search (blastn) using sequences of EST libraries (Yatabe *et al.* 2009).

Marker	Accession no.	Total length (bp)	Coding region (bp)	Non-coding region (bp)	Putative gene (species)	GenBank hit accession no. (E-value)
EST_L058	FS993661	198	101	97	glycolate oxidase (gox) (*Zantedeschia aethiopica*)	AY173074 (2×10^{-124})
EST_L110	FS993713	548	148	400	glycerol-3-phosphate (*Zea mays*)	EU964956 (9×10^{-77})
EST_L258	FS993861	317	79	238	ribosomal protein L17 (*Castanea sativa*)	AF334838 (9×10^{-102})

TABLE 4. Allele types, numbers of clones used, and variations between a consensus sequence and the original clones for *Osmunda ×mildei* samples in three nuclear markers examined.

			Number of clones:				
	Allele	Genbank accession No.	Included in consensus seq.	Identical to consensus seq.	Differing by one subst'n	Differing by two subst'ns	Differing by three subst'ns
EST_L058	A	AB672788	11	7	4	-	-
	B	AB672789	4	4	-	-	-
EST_L110	A	AB672826	10	4	3	1	2
	B	AB672827	4	2	1	-	1
EST_L258	A	AB672863	6	3	3		
	B	AB672864	7	4	1	2	

The *rbcL* sequence of *O. ×mildei* is identical to that of *O. vachellii* and also was similar to those of *O. banksiifolia* and *O. javanica* Blume (Fig. 3). The *Osmunda ×mildei* sequence was more distantly related to *O. angustifolia* (but with low support), and very far from *O. japonica* and other species of subgenus *Osmunda*.

In the three nuclear markers, the *O. ×mildei* sample has two distinct allele types (Figs. 4–6). One type (Type A) had the same sequence as some plants of *O. japonica*, while the other (Type B) had the same sequence as *O. vachellii* (in EST_L58 and L110 in Figs. 4 and 5) or a sequence very similar to it (in EST_L258 in Fig. 6). In each of the three nuclear-marker trees, *O. ×mildei* was more closely related to *O. vachellii* than to *O. angustifolia*.

DISCUSSION

Our trees constructed from the chloroplast gene and nuclear DNA sequences agree with previous trees in the monophyly of the three subgenera of *Osmunda* (Yatabe *et al.*, 1999; Gou *et al.*, 2008; Metzgar *et al.*, 2008). The *rbcL* phylogenetic relationships of the subgenera are the same as Yatabe *et al.*'s (1999) from the same gene and Metzgar *et al.*'s (2008) from seven chloroplast loci including *rbcL*, and different from Gou *et al.* 's (2008) *rbcL* relationships. The relationships deduced from the three nuclear EST markers are not consistent with each other, but the relationship of the EST_L110 agrees with the *rbcL* relationship in the subgenera *Osmunda* and *Plenasium* being sister to each other.

All the phylogenetic trees inferred from the three nuclear EST markers show that *Osmunda ×mildei* has two distinct allele types, and one is identical to those of *O. japonica*, and the other formed a monophyletic clade with those of *Plenasium* species (Figs. 4–6). It is suggested that *O. ×mildei* is an intersubgeneric hybrid between the subgenera *Osmunda* and *Plenasium*. The Type B alleles of *O. ×mildei* have the same EST_L058, EST_L110 sequences and the closest EST_L258 sequences to those of *O. vachellii*, suggesting that *O. ×mildei* is most likely derived by hybridization of *O. japonica* and *O. vachellii*

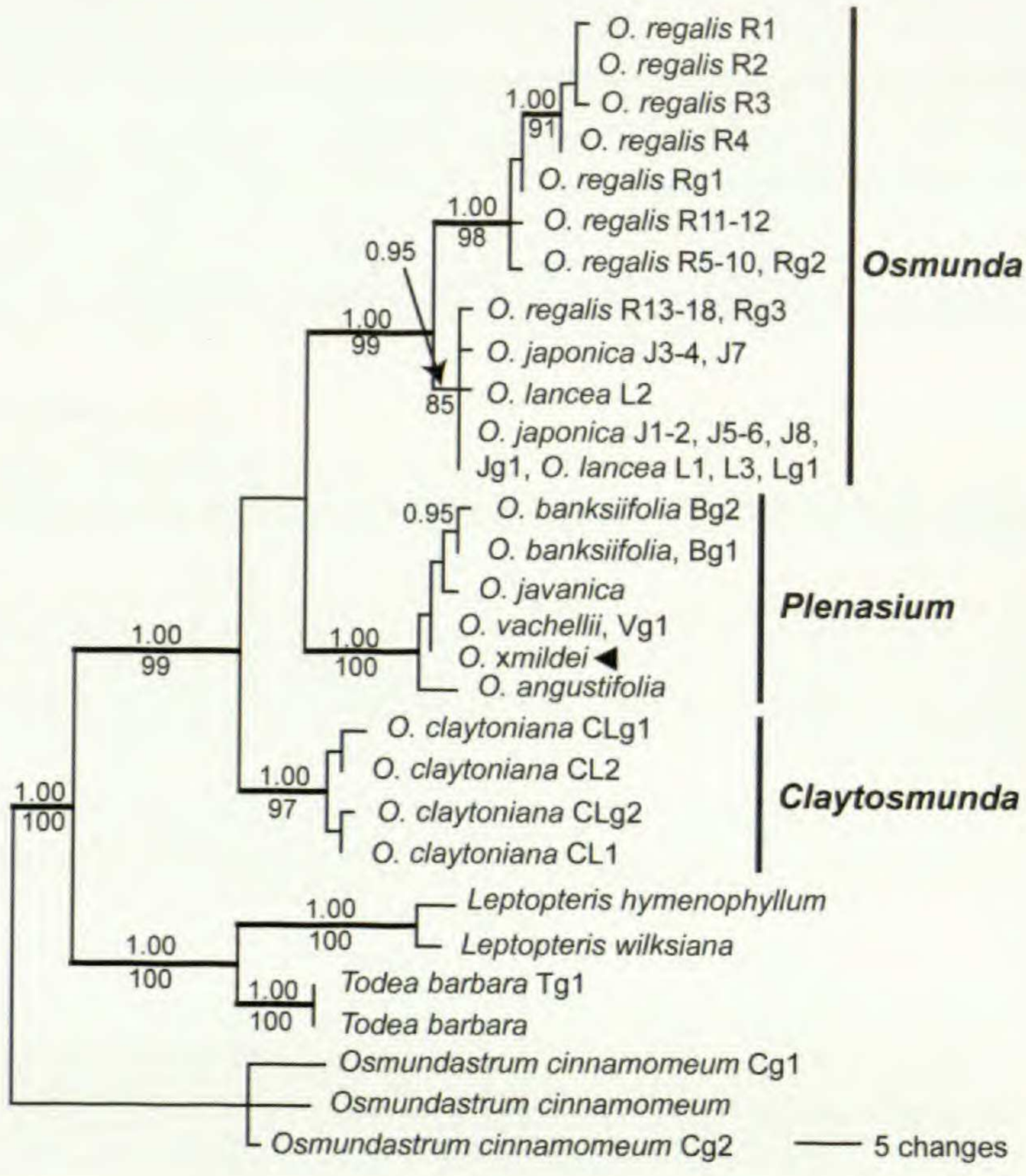

FIG. 3. Bayesian consensus tree based on chloroplast *rbcL* (1227 bp). Values above branches indicate posterior probabilities (>0.9) calculated by Bayesian analysis and those below branches indicate maximum parsimony bootstrap values (>60). Thick branches are highly supported (posterior probabilities p>0.95 and bootstrap values >90). Arrowhead indicates *O.* ×*mildei*. Abbreviations of materials follow Table 1.

(Figs. 4–6). The chloroplast *rbcL* sequence of *O.* ×*mildei* is identical to that of *O. vachellii* (Fig. 3), suggesting that it is the maternal progenitor of *O.* ×*mildei*; hence *O. japonica* is paternal. This suggested parentage agrees with Zhang *et al.* (2008) and Gou *et al.* (2008), who suggested *O. vachellii* as the maternal parent, based on chloroplast DNA data and distributional data. *Osmunda banksiifolia* is also very closely related to *O.* ×*mildei*, however, comparative morphology does not support that *O. banksiifolia* is a parent, because it has prominently dentate pinnae, whereas *O. vachellii* and *O.* ×*mildei* (and also *O. japonica*) are both distinct with entire or somewhat serrate pinnae or pinnasegments.

This study analyzed a sample of *O.* ×*mildei* from Shenzhen, Guangdong (Fig. 2). It has very low spore viability and a very low offspring reproduction rate even in carefully controlled culture conditions (Zhang *et al.*, 2008; J.-F. Yang *et al.*, unpubl. data). Considering the low reproductive ability and a few isolated localities in southern China, it is possible that *O.* ×*mildei* is of multiple origins, although no molecular evidence is available. *Osmunda* ×*ruggii* of eastern North America (Connecticut and Virginia, USA) is an

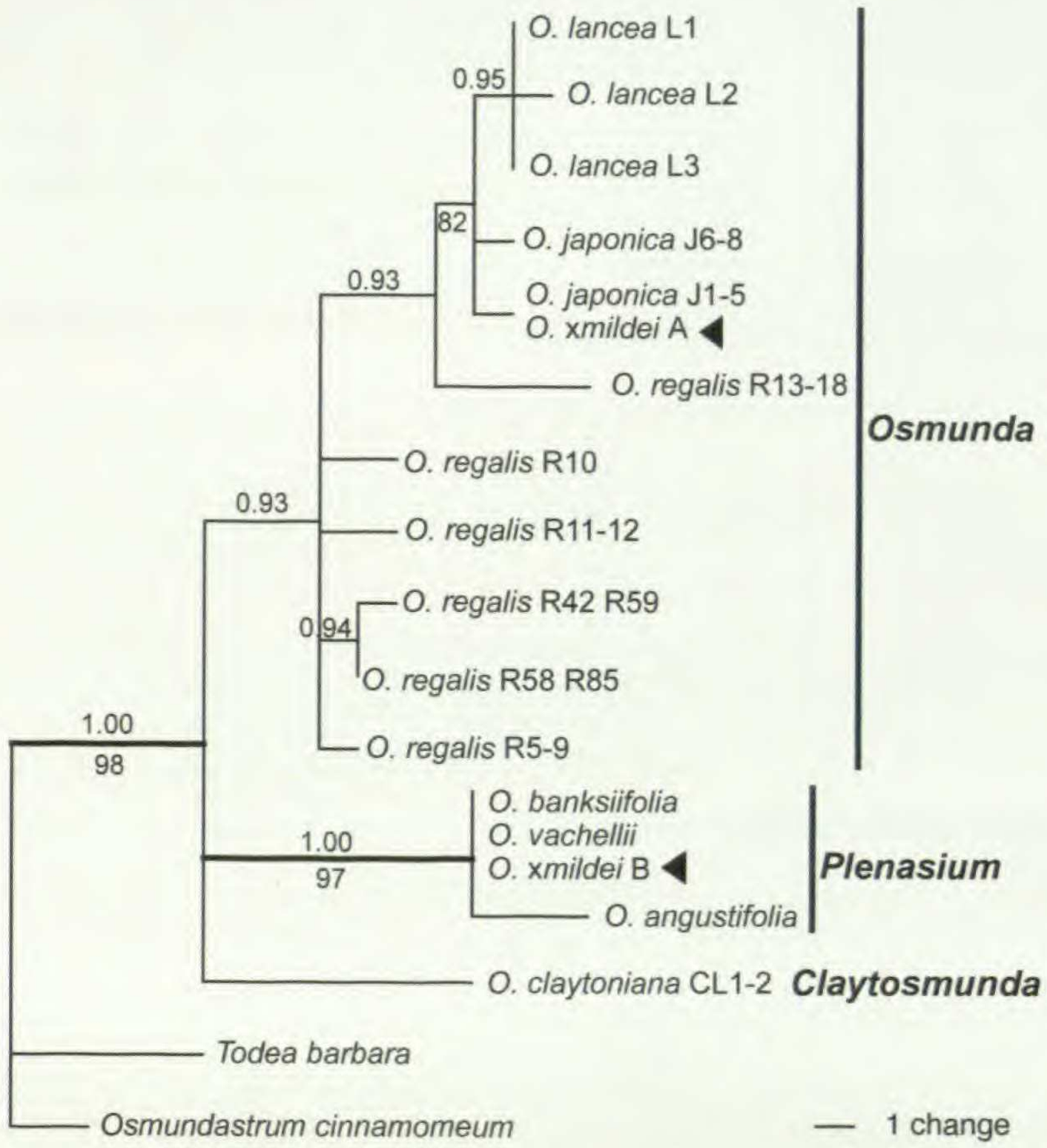

FIG. 4. Bayesian consensus tree based on nuclear EST_L058 (198 bp). Figures above branches indicate posterior probabilities (>0.9) calculated by Bayesian analysis and those below branches indicate maximum parsimony bootstrap values (>60). Thick branches are highly supported (posterior probabilities >0.95 and bootstrap values >90). Abbreviations of materials follow Table 1. Arrowheads indicate allele types obtained from *O.* ×*mildei*. Numbers of clones of each allele type are in Table 4.

intersubgeneric sterile hybrid derived from *O. regalis* (subgenus *Osmunda*) and *O. claytoniana* (subgenus *Claytosmunda*; Tryon, 1940; Wagner *et al.*, 1978; Whetstone and Atkinson, 1993; Li and Haufler, 1994). Like *O.* ×*mildei*, *O.* ×*ruggii* grows together with the parents in a few localities (Wagner *et al.*, 1978). Wagner *et al.* (1978) described that transplants produced fertile pinnae, but produced aborted spores and only univalent chromosomes at meiosis, suggesting its high sterility. *Osmunda* ×*intermedia*, which is widely distributed across Japan, is suggested to be an intrasubgeneric hybrid of multiple origins from *O. japonica* and *O. lancea*, and it is self-reproducible (Shimura, 1972; Yatabe *et al.*, 2009). The differing levels of fertility and sterility in the three hybrids may reflect close or remote phylogenetic affinities (Yatabe *et al.*, 1999; Metzgar *et al.*, 2008).

Osmunda japonica is distributed in eastern Asia extending west to the Himalayas and south to northern Vietnam (Kato, 2007). It is a likely parent for *O.* ×*mildei*, *O.* ×*nipponica* and *O.* ×*intermedia*, all three of which occur within the distributional range of *O. japonica*. *Osmunda vachellii*, the other parent for *O.* ×*mildei*, also co-occurs with *O.* ×*mildei* (Fig. 2). From this

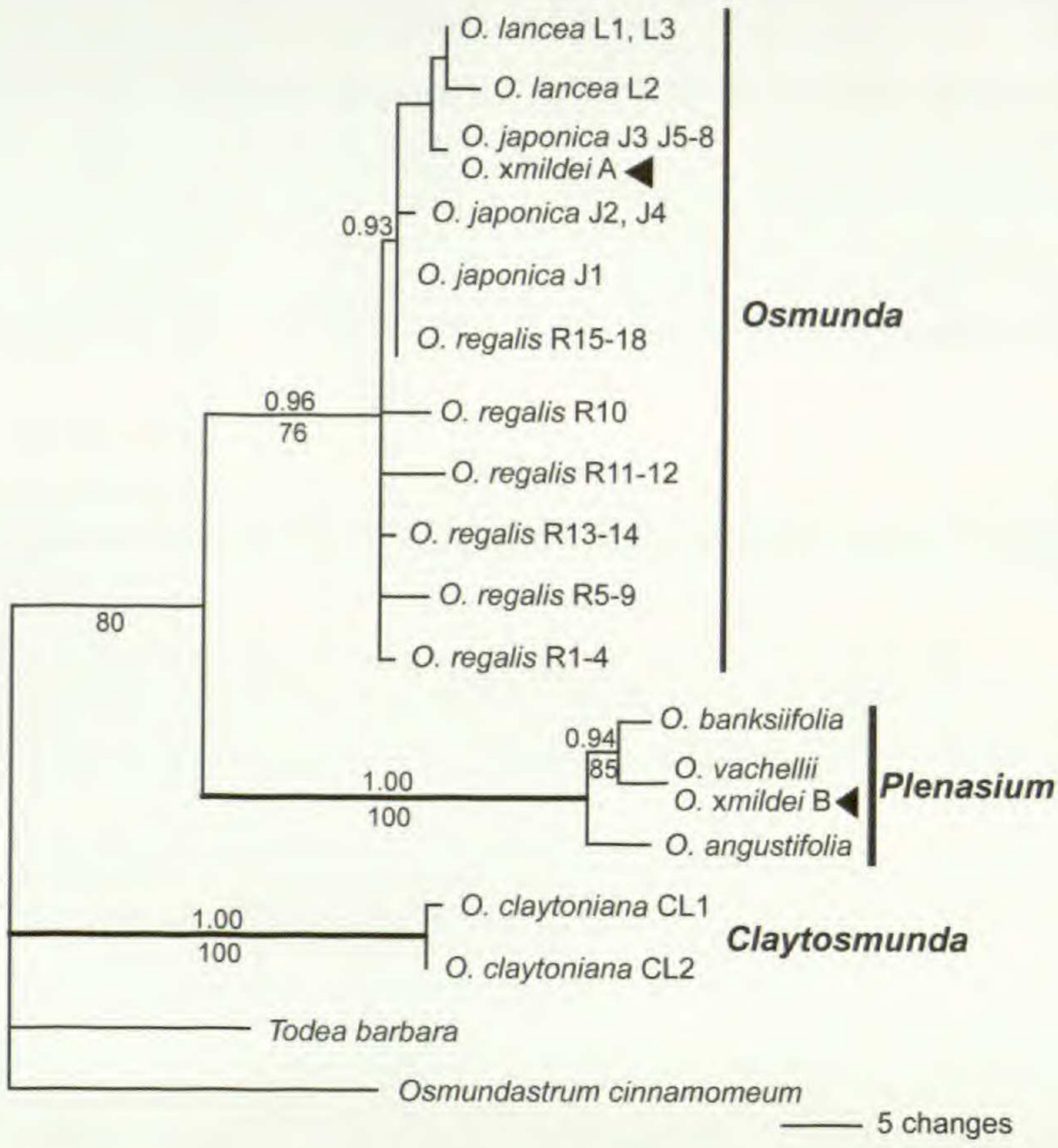

FIG. 5. Bayesian consensus tree based on nuclear EST_L110 (572 bp). Values above branches indicate posterior probabilities (>0.9) calculated by Bayesian analysis and those below branches indicate maximum parsimony bootstrap values (>60). Thick branches are highly supported (posterior probabilities >0.95 and bootstrap values >90). Abbreviations of materials follow Table 1. Arrowheads indicate allele types obtained from *O.* ×*mildei*. Numbers of clones of each allele type are in Table 4.

distributional pattern, along with the distributions of *O.* ×*ruggii* and its parents, we suggest that overlap of the parental species allowed the interspecific hybridization relatively recently. On the contrary, a fertile tetraploid species of hybrid origin between *O. japonica* and *O. regalis* (subgenus *Osmunda*), occurs in northern Central Laos distant from the distribution ranges of both parents, and in northern Myanmar, distant from Central India where *O. regalis* occurs (Kato, 2007; Tsutsumi *et al.*, 2011). Tsutsumi *et al.* (2011) suggested that the hybrid species arose when the distribution ranges of the parents overlapped, a pattern different from the current pattern.

ACKNOWLEDGMENTS

We thank S. Akiyama, M. K. Bhattacharya, W.-L. Chiou, A. Ebihara, C. R. Fraser-Jenkins, P. Hovenkamp, N. Katayama, S. Kobayashi, S. Koi, G. Kokubugata, L.-Y. Kuo, T. Minamitani, R. C. Monica, R. Moran, H. Murata, J. Murata, T. Nakamura, H. P. Nooteboom, T. Oka, A. K. Pradeep, S. Suddee, A. Tuji, T. Wongprasert, G. Yatskievych, X.-C. Zhang, and M. Zink for their help during the trips or providing materials. We also thank H. Liu, Shenzhen Fairylake Botanical Garden for

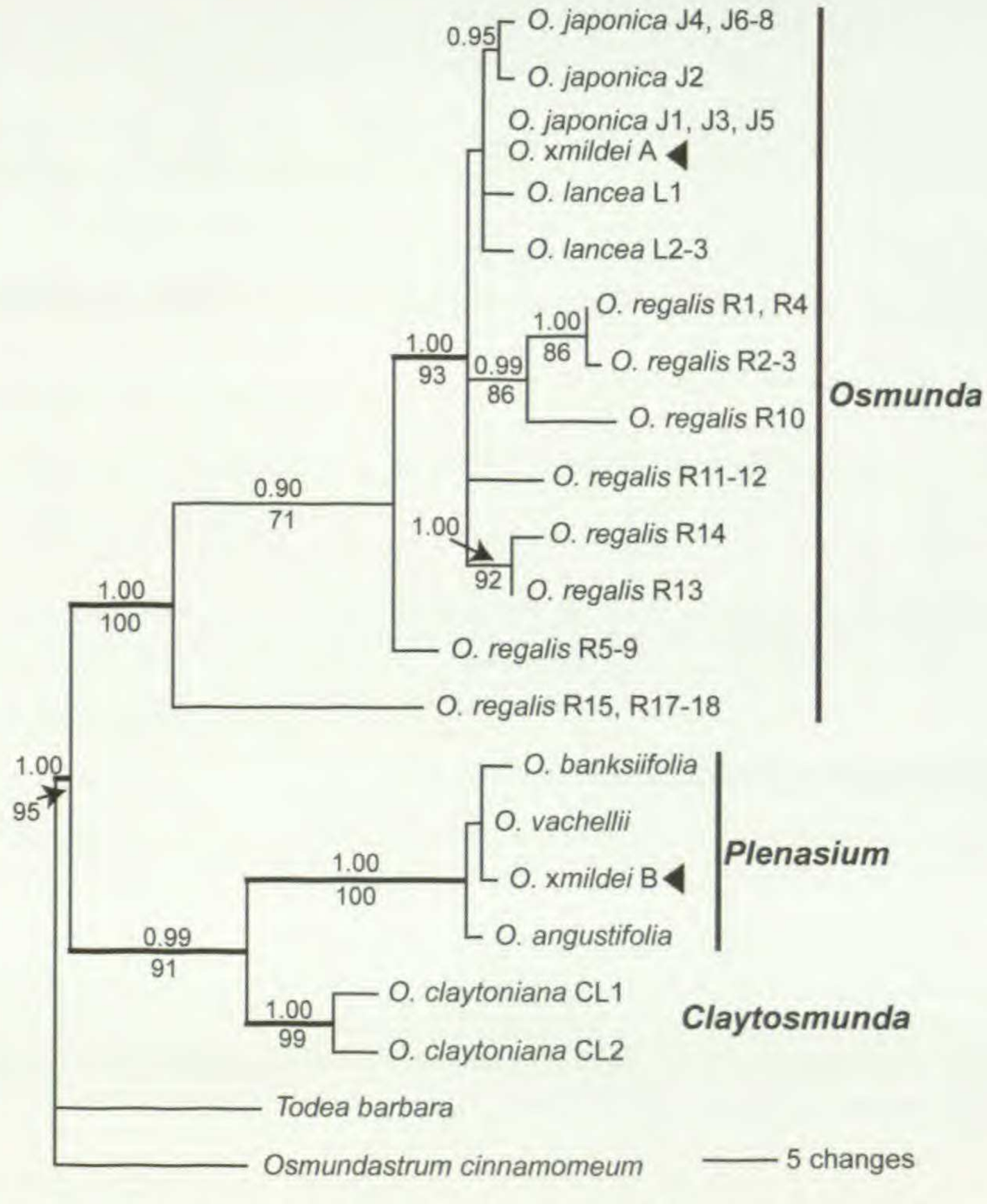

FIG. 6. Bayesian consensus tree based on nuclear EST_L258 (361 bp). Values above branches indicate posterior probabilities (>0.9) calculated by Bayesian analysis and those below branches indicate maximum parsimony bootstrap values (>60). Thick branches are highly supported (posterior probabilities p>0.95 and bootstrap values >90). Abbreviations of materials follow Table 1. Arrowheads indicate allele types obtained from *O.* ×*mildei*. Numbers of clones of each allele type are in Table 4.

sending a copy of a reference cited here and M. Nakajima for the illustrations. This study was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.

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